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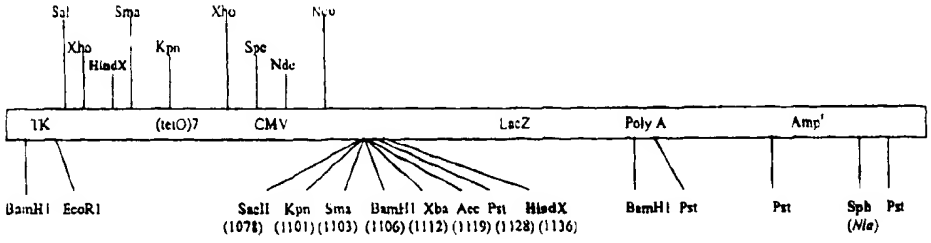

(57) Abstract

A control system for genetic expression includes a first, reporter polynucleotide molecule including a reporter sequence encoding a protein to be expressed operably linked to a heptameric tet operator sequence and at least two promoters and a second, regulator polynucleotide molecule including a sequence coding for a tet repressor protein and the KRAB protein. The reporter polynucleotide includes one promoter on each side of the tet operator sequence. In one embodiment a TK promoter is located upstream of the operator sequence and a CMV promoter is located downstream thereof. The desired nucleic acid sequence of the reporter molecule is positioned downstream of the CMV promoter. A method to regulate the expression of a protein using the system of the invention is provided. The invention also provides a kit including both the polynucleotides described above.

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SYSTEM AND METHOD FOR REGULATION OF GENE EXPRESSION

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to the field of gene expression. More specifically, the invention relates to a system and method for regulating gene expression.

DESCRIPTION OF THE PRIOR ART

In studying gene function, various control systems have been developed to aid in analysing the steps involved in genetic expression. Some of these control systems comprise "switches" for turning on or off the expression of certain genes. However, switches developed to date have associated therewith various drawbacks. For example, although some of these switches, classified as "on" switches, induce high levels of expression of a desired protein, the basal level of expression in the "off" state is still relatively high. Other such switch systems, classified as "off" switches, reduce basal expression levels; however, the levels of induction when switched "on" are not high. Specific examples of such systems are provided below. In other systems, the triggers for the switches have deleterious effects on the host cell.

One system that has been under extensive investigation has been the lactose operon which codes for the β-galactosidase gene. The bacterial β-galactosidase gene (*lacZ*) is an excellent reporter gene and is used extensively in life science applications including: cloning (Sambrook et al, 1989, *Molecular Cloning: A Laboratory Manual,* 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, N.Y.); promoter assessment (Park et al, 1994, "A β-Galactosidase Expression Vector For Promoter Analysis", *DNA and Cell Biology*, 13:1147-1149); gene regulation and function; and mutation analysis (Gossen et al, 1989, "Efficient Rescue Of Integrated Shuttle Vectors From Transgenic Mice: A Model For Studying Mutations In Vivo", *Proc. Natl. Acad. Sci.*, 86: 7981-7985). The contents of these references are incorporated herein by reference. The *lacZ* gene provides straightforward results regarding expression and function upon chromogenic assays using the substrate X-gal or ONPG; the quantitation of the protein is easily and reliably attained upon cell lysis, staining

and spectrophotometric analysis; and the detection of in-vivo expression of the *lacZ* in mammalian cells is easily accomplished using basic histochemical staining.

It is of interest to have a reporter whereby transcription may be experimentally controlled. Although many eukaryotic promoters have been identified and cloned, they are, as mentioned above, often leaky (i.e., they do not provide total on-off control) and/or lack responsiveness in mammalian cell lines. Moreover, also as mentioned above, the inducers are generally deleterious to the host cell or have significant drawbacks such as toxicity.

Many chimeric transcription factors have been developed in order to ameliorate these problems. Earlier attempts at producing chimeric transcription factors were based on using the glucocorticoid receptor (Baniahmad et al, 1993, "Mechanisms Of Transcriptional Activation By Steroid Hormone Receptors", *J. Cell. Biochem.*, 51: 151-6). More recently the bacterial *lac* and *tet* operons have been exploited. The *lac* based systems have been characterised as "leaky" whereas the tet-based binary systems have exhibited greater fidelity between protein and operator sequences. Additionally, the tet-based systems are inducible using tetracycline, a well known and well characterised compound.

Two tetracycline responsive binary systems have been previously described (Deuschle et al, 1995, "Tetracycline-Reversible Silencing Of Eukaryotic Promoters", *Mol. and Cell. Biology*, 15: 1907-1914; and, Gossen and Bujard, 1992, "Tight Control Of Gene Expression In Mammalian Cells By Tetracycline-Responsive Promoters", *Proc. Natl. Acad. Sci. U.S.A.*, 89: 5547-5551 and in U.S. patent 5,464,758 (1995). The contents of theses documents are incorporated herein by reference. A binary system comprises two plasmids: a regulator plasmid and a reporter plasmid. The regulator plasmid expresses a protein which, under certain conditions, activates transcription of the reporter plasmid. The reporter plasmid, in turn, codes for a reporter protein. Both the Deuschle (1995) and the Gossen and Bujard (1992) systems include a reporter plasmid having a *cis* regulatory sequence composed of a heptameric repeat of the tetracycline operator (tetO), which is a 19 bp inverted repeat, fused to the immediate early sequences of the cytomegalovirus enhancer (CMV) to provide a plasmid including a sequence represented as (tetO)7-CMV. This sequence is located upstream of the reporter gene. However, the chimeric transcription factors, or the regulator plasmids, are quite different in both these references. The tetracycline-controlled transactivator (tTA) system of Gossen and Bujard is composed of the tetR, tetracycline repressor protein fused to the transactivating carboxy terminus of the virion protein 16, VP-16, of the herpes simplex virus to result in the tetR-VP16 complex. The chimeric protein

described by Deuschle consists of the highly conserved KRAB (Kruppel-associated box) domain of the Kox1 Zinc finger protein family fused to the tetR protein to result in tetR-KRAB. In both references, a CMV promoter is provided in the regulator plasmids, upstream of the tetR sequence, to ensure constitutive expression of the regulator protein.

These two motifs differ in their kinetics and mechanism of protein-DNA associations. The tTA (i.e. tetR-VP16) system strongly induces expression of the reporter gene in the absence of tetracycline. In the uninduced state, however, basal levels of expression tend to be relatively high due to the CMV sequences which also form part of the system. The tetR-KRAB system makes use of the transcriptional silencing influence of the KRAB domain. This system maintains much lower levels of basal activity in the repressed state and is induced by the addition of tetracycline. However, induction levels using this system are much lower than that observed with the tTA system.

Thus, a new control system is desired which overcomes the disadvantages of the known gene expression regulatory systems and which provides a system which maintains a low level of basal expression when in the off state and high levels of induction when in the on state.

SUMMARY OF THE INVENTION

Accordingly, in one aspect, the present invention provides a polynucleotide molecule including a desired nucleic acid sequence operably linked to an operator sequence and at least two promoters.

In another aspect, the present invention provides a eukaryotic cell transfected with:

a first polynucleotide molecule including a desired nucleic acid sequence operably linked to a tet operator sequence and at least two promoters; and,

a second polynucleotide molecule coding for a transrepressor fusion protein comprising a tet repressor and a transcription silencer protein.

In yet another aspect, the present invention provides a kit comprising at least two containers wherein the first container contains a first polynucleotide molecule including a desired nucleic acid sequence operably linked to a tet operator sequence and at least two promoters and wherein the second container contains a second polynucleotide molecule including a gene for a tet repressor protein (tetR) and the Kruppel-associated box domain (KRAB) of Kox1 Zn-finger protein.

BRIEF DESCRIPTION OF THE DRAWINGS

The features of the preferred embodiments of the invention will become more apparent in the following detailed description in which reference is made to the appended drawings wherein:

Figure 1 is a restriction map of an operator sequence of a reporter plasmid in accordance with a preferred embodiment;

Figures 2a and 2b are a representation of the cloning of the operator sequence of Figure 1;

Figure 3 shows the tetracycline-responsiveness of transiently transfected HeLa cells.

Figures 4a and 4b are a representation of the cloning of the operator sequence of Figure 1 into a λ phage.

Figure 5 illustrates the polynucleotide sequence (SEQ ID NO: 1) of the upstream primer 2HU used in the PCR amplification of a portion of the reporter plasmid of the invention.

Figure 6 illustrates the polynucleotide sequence (SEQ ID NO: 2) of the downstream primer 1HD used in the PCR amplification of a portion of the reporter plasmid of the invention.

Figure 7 illustrates the polynucleotide sequence (SEQ ID NO: 3) of the upstream primer 1HTKU used in the PCR amplification of the TK-(tetO)7-CMV-*lacZ* sequence of the invention.

Figure 8 illustrates the polynucleotide sequence (SEQ ID NO: 4) of the upstream primer 1HLacD used in the PCR amplification of the TK-(tetO)7-CMV-*lacZ* sequence of the invention.

Figure 9 is a photograph of the results from a gel electrophoresis of the PCR reactions amplifying the (tetO)7-CMV sequence of the reporter plasmid.

Figure 10 is a photograph of the results from a gel electrophoresis of the Long PCR reactions amplifying the Tk-(tetO)7-CMV-*lacZ* sequence for cloning into the λgt10 phage.

Figure 11 illustrates the polynucleotide sequence (SEQ ID NO: 5) of the upstream primer 3HU used in the PCR amplification and characterization of the TK-(tetO)7-CMV-*lacZ* sequence of the invention.

Figure 12 is a representation of the PCR amplifications used in characterizing the TK-(tetO)7-CMV-*lacZ* sequence of the invention.

Figure 13 illustrates the polynucleotide sequence (SEQ ID NO: 6) of the downstream primer MB used in the PCR amplification and characterization of the TK-(tetO)7-CMV-*lacZ* sequence of the invention.

Figure 14 illustrates the polynucleotide sequence (SEQ ID NO: 7) of the upstream primer ME used in the PCR amplification and characterization of the TK-(tetO)7-CMV-*lacZ* sequence of the invention.

Figure 15 is a photograph of the results from a gel electrophoresis of the PCR amplification and characterization of the TK-(tetO)7-CMV-*lacZ* sequence of the invention.

Figure 16 is a photograph of the results from a gel electrophoresis of the PstI digests of the PCR products of the TK-(tetO)7-CMV-*lacZ* sequence of the invention.

Figure 17 is a photograph of the results from a gel electrophoresis of the endonuclease reactions of the p(TK-*lacZ*) and p(TK-(tetO)7-CMV-*lacZ*) plasmids of the invention.

Figure 18 illustrates the tetracycline-responsiveness of transiently transfected NIH 3T3 cells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the following description, a number of recombinant DNA technology terms are used. The following definitions have been provided in order to provide a clearer understanding of the specification and appended claims:

"Promoter" - a DNA sequence generally described as the 5' region of a gene, located proximal to the start codon. The transcription of an adjacent gene is initiated at the promoter region. If a promoter is an inducible promoter then the rate of transcription increases in response to an inducing agent.

"Minimal Promoter" - a partial promoter sequence which defines the transcription start site but which by itself is not capable of initiating transcription efficiently. The activity of minimal promoters depends on the binding of activators to operably linked binding sites.

"Operably Linked" - a nucleic acid sequence is "operably linked" when placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is "operably linked" to a coding sequence if the promoter causes the transcription of the sequence. Generally, operably linked means that the linked nucleic acid sequences are contiguous and, where it is necessary to join two protein coding regions, contiguous and in one reading frame.

"Gene" - a DNA sequence that contains information needed for expressing a polypeptide or protein.

"Reporter Gene" – a gene used to indicate functional expression and/or which is easily identified using conventional detection methods.

"Polynucleotide Molecule" - a polydeoxyribonucleic (DNA) acid molecule or a polyribonucleic acid (RNA) molecule.

"Expression" - the process by which a polypeptide is produced from a structural gene.

"Tetracycline Analogue" - any one of a number of compounds that are closely related to tetracycline and which bind to the tet repressor (tetR).

In one embodiment, the present invention provides a polynucleotide molecule including a sequence for a desired cellular nucleic acid, the transcription of which is controlled by the presence of tetracycline. The desired cellular nucleic acid may comprise a gene for a structural or reporter protein, RNA or any other component. The polynucleotide molecule includes a sequence for the desired cellular nucleic acid operably linked to a tet operator sequence and at least two promoters. In the preferred embodiment, one promoter sequence is provided upstream and downstream of the operator sequence. In a preferred embodiment, the polynucleotide molecule includes a regulatory *cis* element comprising an operator sequence with a heptomeric repeat of the tetO operator and represented as (tetO)7. The tetO operator is derived from the tetracycline-resistance operon encoded in the Tn10 transposon of *E. coli*. On the upstream side of the (tetO)7 operator sequence is provided a thymidine kinase minimal promoter (TK) of the herpes simplex virus. On the downstream side of the (tetO)7 sequence is provided an immediate early promoter sequence from the cytomegalovirus (CMV). Although CMV is a strong promoter, it is also, and usually, referred to as an "enhancer". Enhancers are sequences capable of influencing the expression of genes over very long distances, for example up to 40 Kb, unlike typical promoters such as TK or SV.

Downstream of the CMV promoter sequence is provided the sequence for the desired protein. In one embodiment, the protein sequence comprises the nucleotide sequence coding for β-galactosidase and represented as *lacZ*. Thus, the polynucleotide molecule in the preferred embodiment is represented as TK-(tetO)7-CMV-*lacZ* and is schematically illustrated in Figures 1 and 4a which also indicate the restriction sites included in the construct. It will be understood by persons skilled in the art that *lacZ* serves as a reporter

gene for the purposes of illustrating the present invention and that any gene, RNA or other biochemical product encoded by nucleic acids may be utilized including reporter and structural genes. Indeed, in another embodiment, the invention includes uses of the polynucleotide sequences described herein to regulate the expression of structural genes in cells either in-vitro or in-vivo. In addition, the invention may be applied to control an RNA sense or anti-sense sequence or any other biochemical product encoded by nucleic acids.

Transcription of the TK-(tetO)7-CMV-*lacZ* sequence in a plasmid is tetracycline-responsive and requires the Tet repressor, tetR, for activation.

In binary systems such as those described by Deuschle (1995) and Gossen (1992), two plasmids are provided. Similarly, in another embodiment, the present invention provides for a two plasmid system including a reporter plasmid p(TK-(tetO)7-CMV-*lacZ*) and a regulator plasmid which codes for the chimeric *trans*-repressing protein tetR. The regulator plasmid is also provided with a CMV promoter upstream of the tetR sequence and a downstream transcriptional activator protein domain. In the preferred embodiment, such transactivator comprises the KRAB protein as described by Deuschle (1995) and as described above. Accordingly, in the preferred embodiment, the regulator plasmid may be represented as p(CMV-tetR-KRAB).

In the absence of tetracycline (Tc), the tetR-KRAB protein expressed by the regulator plasmid activates transcription of the reporter plasmid thereby resulting in expression of the reporter gene, *lacZ*. Upon addition of Tc, the tetR-KRAB protein-(tetO) sequence interaction is destabilized and transcription of the reporter plasmid is halted. As mentioned above, the silencing effect of the KRAB domain of the regulator plasmid ensures that basal expression of the reporter plasmid is maintained at low levels and, therefore, provides an effective "off" switch for transcription of the reporter plasmid in the presence of tetracycline.

In another embodiment, the transcriptional activator of the regulator plasmid may code for the VP16 protein as described by Gossen (1992). The regulator plasmid according to such embodiment may be represented as p(tetR-VP16). Similarly, in another embodiment, the invention provides a regulator plasmid encoding the tetR domain fused to other commonly known transactivator domain.

In another embodiment, the invention provides for vectors containing the reporter and regulator nucleotide sequences of the two plasmids described above. The recombining of the sequences of the invention into such vectors may be accomplished by any conventional technique. A preferred embodiment is described below.

Construction of the p(TK-(tetO)7-CMV-*LacZ)* Reporter Plasmid

The reporter plasmid of the invention was constructed in accordance with the following methodology.

The pCH110 plasmid (Pharmacia Biotech Inc., Piscataway, NJ), including the *lacZ* sequence, was modified as described in Park et al (1994) referred to above, the contents of which are incorporated herein by reference. The pCH110 plasmid is derived from the pBR322 plasmid. The modification referred to above results in an addition, upstream of the *lacZ* sequence, of a TK minimal promoter and multiple cloning sites (MCS) to result in a ~7.1 Kb plasmid represented as p(TK-*lacZ*) referred to in this study as the parent vector. This plasmid is schematically illustrated in Figure 2b. The MCS sequence of the plasmid contains the following restriction sites in order: Sac, Kpn, Sma, BamH1, Xba, Acc, Pst1.

The 5.79 Kb Deuschle plasmid, p(tetO)7-CMV-*luc*, schematically illustrated in Figure 2a, was used as the template in a polymerase chain reaction (PCR) in order to obtain the required sequences. The PCR reaction is schematically illustrated in Figures 2a and 2b. For the PCR reaction, the upstream primer comprised the sequence identified as SEQ ID NO:1 and as shown in Figure 5 and referred to as 2HU in Figure 2a. The 2HU primer introduces a *HindIII* restriction site, as shown underlined in Figure 5, which is inactivated after ligation (as described below). The downstream primer, referred to as 1HD, comprises the sequence identified as SEQ ID NO:2 and is illustrated in Figure 6. Primer 1HD incorporates the *HindIII* site as shown underlined in Figure 6.

Because the upstream *HindIII* site was introduced, the annealing temperature was lowered for the first 6 rounds of PCR. PCR profiles were as follows: 94°C, 30 sec; 59°C, 1.5 min; 72°C, 30 sec for 6 rounds followed by 25 rounds with the standard PCR profile 94°C, 62°C, 72°C for 30 sec, 1.5 min, 30 sec respectively.

As illustrated in Figures 2a and 2b, the PCR reaction described above results in preferential amplification of the PCR products (~1.16Kb) comprising the sequence (tetO)7-CMV flanked by *HindIII* sequences. The PCR products were then digested with HindIII. The digested products were then ligated into the parent plasmid, p(TK-*lacZ*), at the *HindIII* site of the MCS sequence. Such ligation results in the ~8 Kb reporter plasmid according to a preferred embodiment of the invention: p(TK-(tetO)7-CMV-*lacZ*) as shown in Figure 1 and Figure 4a.

Figure 9 shows a photograph of a gel electrophoresis of the amplified products from the above PCR reaction using the p(tetO)7-CMV-*luc* plasmid as the template.

In order to confirm the presence of the CMV, (tetO)7 and TK sequences in the reporter plasmid, the plasmid was subjected to a series of PCR reactions using the primers 3HU, MB, and ME as schematically illustrated in Figure 12. These plasmids have sequences respectively identified as SEQ ID NO: 5, SEQ ID NO: 6 and SEQ ID NO: 7 and are shown in Figures 11, 13 and 14, respectively. In the first step, the PCR was conducted using the primers 3HU and MB and subsequently digested with Xho. In the next step, the plasmid was PCR'd with the primers ME and MB and also digested with Xho. Both digests were put through a gel electrophoresis and a photograph of the results are shown in Figure 16. Lane 1 contains the digest products from the first step while Lanes 2 and 3 contain the digest products from the second step. The standard is indicated in the last lane as "S".

In Figure 17, the results from a PstI digest of the reporter plasmid p(TK-(tetO)7-CMV-*lacZ*) is illustrated. In Lanes 2, 3, 5, and 6 are results from the digestion of purified plasmid with the restriction endonuclease PstI. Lanes 7 to 10 and 12 are undigested purified plasmids. Lanes 1, 3 and 11 are molecular weight standards, 1Kb ladder (Gibco BRL). Lanes 2 and 7 are the plasmid p(TK-(tetO)7-CMV-*lacZ*). Lanes 3 and 8 are plasmid p(TK-*lacZ*) and the parent vector. Lane 10 is the plasmid p(*lacZ*). The results from this study confirm the sequence orientation of the reporter plasmid.

Figure 18 shows the results from the digestion of the plasmids p(TK-(tetO)7-CMV-*lacZ*) and p(TK-*lacZ*) with restriction endonuclease. The plasmid p(TK-(tetO)7-CMV-*lacZ*) was loaded in Lanes 3, 6, and 9. Lanes 2, 5, and 8 contained the p(TK-*lacZ*) plasmid. Lanes 1, 4, 7, and 10 contained 1Kb molecular weight standards (Gibco BRL). These results confirm the sequence orientation of the reporter plasmid p(TK-(tetO)7-CMV-*lacZ*).

Cloning of the Reporter Plasmid Into λ Phage Vectors

Long PCR of the reporter plasmid p(TK-(tetO)7-CMV-*lacZ*) was conducted using primers referred to as 1HTKU and 1HLacD. Primer 1HTKU, having the sequence shown in SEQ ID NO:3 and as shown in Figure 7, incorporates a *HindIII* site and recognizes a sequence upstream (5') of the TK promoter of the reporter plasmid. Primer 1HLacD, having the sequence shown in SEQ ID NO:4 and as shown in Figure 8, also incorporates a *HindIII* site and recognizes a sequence downstream of a SV-40 polyadenylation site on the reporter plasmid.

PCR reactions were conducted as follows: denaturing at 93°C for 30 seconds, annealing at 62°C for 2.5 minutes, and elongation at 65°C for 5 minutes. A 15 second extension was added to each elongation cycle. PCR reactions proceeded to 40 cycles in a Perkin Elmer thermal cycler using the rTth DNA polymerase and the XL buffer pack (Perkin Elmer). The products from the PCR reaction comprised the TK-(tetO)7-CMV-*lacZ* sequence flanked by *HindIII* sticky ends.

PCR reactions with nested primers and restriction endonucleases with agarose gel electrophoresis was used to screen and confirm the Long PCR products as containing the TK-(tetO)7-CMV-*lacZ* sequence. Figure 10 shows a photograph of the gel electrophoresis of the amplified products of the above Long PCR reaction. In the photograph: Lanes 1 and 2 contain negative controls. Lane 9 contains positive control template pTKLacZ. Lanes 3 – 7 are reactions using upstream primer 3HU (SEQ ID NO:5, and shown in Figure 11), resulting in products that do not contain the (tetO)7-CMV chimera sequences. Lanes 10 – 13 are from reactions using the 1HTKU upstream primer. Both sets of reactions use a common downstream primer 1HLacD. Lanes 8 and 14 contain the 1KB molecular weight marker (Gibco BRL).

The products of the above PCR reaction were blunt end filled and ligated into an EcoR1 site of the phage λgt10 as described below. The PCR reaction and ligation steps are schematically represented in Figures 4a and 4b. The EcoR1 CIP treated λgt10 vector was purchased from Stratagene (La Jolla, CA, USA). The TK-(tetO)7-CMV-*lacZ* sequence was purified using column chromatography and was subsequently blunt-end ligated into Klenow filled λgt10. Ligation products were purified using CsCl. Phage DNA was packaged and plated on *E.coli* C bacteria. Twelve putative clones (plaques) were cored and soaked in SM buffer. Clones (including two negatives) were then screened using PCR with nested primers. Positives were then prepared using standard methods (as per Sambrook et al (1989), incorporated herein by reference), large scale λ liquid lysis.

The phage construct is referred to herein as λ(TK-(tetO)7-CMV-*lacZ*). The λ construct can be applied in various ways including:

1) the generation of transgenic animals via microinjection of CBA X C57BL/6.

2) the genereation of cell lines via lipofection of NIH 3T3 cells (described below).

HeLa Cell Culture and Lipofection

HeLa cell lines were cultured in Iscove's modified Dulbecco's Minimal Eagles Medium (DMEM) (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5 % fetal bovine serum (Summit Biotechnology, Fort Collins, CO), penicillin-streptomycin (Life Technologies, Inc.) and fungizone. Cells were seeded at roughly 11.5 x 10^5 cells per 60mm petri dish and were incubated overnight to roughly 70-80% confluency. DNA used in the transfection experiments consisted of long PCR products of the p(TK-(tetO)7-CMV-*lacZ*) using primers 1HTKU and 1HLacD or purified plasmids from heat shock transformed DH5αMaxEfficiency hosts (Life Technologies, Inc., Gaithersburg, MD), using the Qiagen midi prep protocol (Qiagen Inc., Chatsworth, VA). DNA was transfected using Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) and a 4:1 ratio of the regulator plasmid p(CMV-tetR-KRAB) to the reporter plasmid p(TK-(tetO)7-CMV-*lacZ*). Roughly 2.5 μg of DNA was combined with serum free media to a volume of 160 μL which was then combined with 7-9 μL of Lipofectamine in 160 μL serum free media. Approximately 2 μg of the SV-40 driven β-galactosidase expression vector pCH110 (Pharmacia Biotech Inc., Piscataway, NJ) was used as a positive control. The cultures were washed and provided with fresh media 4-5 hours after transfection. In order to induce expression, media containing sub-inhibitory levels of tetracycline (0.5 μg/ml) was added to two of three cultures (+Tc) while one culture was maintained without tetracycline (-Tc). The tetracycline responsiveness of the transiently transfected HeLa cells after 6 hours and 22 hours after addition of tetracycline (Tc) are illustrated in Figure 3. As can be seen, the addition of tetracycline greatly induces enzyme activity while in the absence of tetracycline, the basal expression level is barely noticeable.

As further shown in Figure 3, high levels of induction are reached within 22 hours after addition of tetracycline. Levels of induction are in the range of 50-300+ fold usually by hour 19 (data not shown). In one particular experiment extremely high levels of induction (>100 fold) were reached within 9.5 hours after transfection. In most cases however, by the 6^{th} to 9^{th} hours, induction levels are within the range of 10-fold.

Assay for β-galactosidase Activity In Transfected HeLa Cells

β-galactosidase activity was measured using the β-galactosidase assay kit (Stratagene, La Jolla, CA) and o-nitrophenyl-β-D-galactopyranoside (ONPG). Briefly, the transfected cells were washed with phosphate buffered saline (PBS) and lysed with a mild detergent. The

supernatants were collected after centrifuging. Cell extracts were analysed by spectrophotometric measurements, OD_{420}. All samples were normalised using Lowry staining (Bio-Rad, Hercules, CA). In order to reduce endogenous mammalian β-galactosidase, samples were soaked at 44 °C for 50 minutes before addition of ONPG.

Transfection of NIH 3T3 Cells

Murine NIH 3T3 cells, derived from one culture, were seeded in 60 mm diameter petri plates, transfected at 80% confluence and were not split throughout the experiment. Cells were transiently co-transfected with a molar ratio of 1.5 p(tetR-KRAB) to p(TK-(tetO)7-CMV-*lacZ*) expression vector. Tetracycline (0.5μg/ml) was added to four of six transfected cultures while two cultures were maintained without tetracycline and tow additional cultures were "mock" transfected. The results of these trials are illustrated in Figure 19. As observed, enzyme expressions levels in the induced state are high while basal expression levels in the un-induced state are relatively low. It can be further seen in Figure 19 that the lower ration of regulator to reporter plasmids (i.e. 1.5:1 vs. 4:1 from Figure 3) leads to a different ratio of enzyme expression. Therefore, by adjusting the ratio of the two plasmids the level of expression control can be tailored to required values.

Binary System For Transcriptional Control

As mentioned above, in one embodiment of the invention, a binary system is provided which includes the p(TK-(tetO)7-CMV-*LacZ*) reporter plasmid as described above and a regulator plasmid including the CMV-tetR-KRAB sequence. Such binary systems are described by Deuschle and Gossen as discussed above. The responsiveness of the transcriptional product of the regulator plasmid provides a tight control on transcription of the reporter plasmid.

In a preferred embodiment, the regulator plasmid includes a T-antigen nuclear localization sequence (NLS), as known in the art, fused to the tetR sequence. The NLS is inserted between the tetR and KRAB sequences and assists the expressed repressor protein in entering the nucleus of the cell.

Discussion

The prior art binary system described by Deuschle, utilizing a CMV-tetR-KRAB regulator plasmid, is extremely reliable in repressing the activity of the reporter plasmid but is

less inducible when compared to the tTA binary system described by Gossen and Bujard (both references being incorporated herein by reference). By constructing a reporter plasmid having a TK-(tetO)7-CMV-*lacZ* sequence (or including any other reporter or structural gene sequence), the present inventors have found that, in the repressed state, TK promotion appears to be inhibited by the bulky tetR-KRAB complex. However, when the repressor-operator interaction is destabilised by the addition of tetracycline, thereby inducing transcription of the reporter plasmid, the TK and CMV promoters of the reporter plasmid have been found to display synergism. In transiently transfected HeLa cells, induction has been shown to be well in excess of 100 fold.

By combining the TK and CMV promoters in the reporter plasmid and utilizing the tetR-KRAB regulator plasmid as described above, the system and method of the present invention provide: i) a much quicker response to tetracycline addition; and ii) a much higher level of induction (by as much as 25 times that observed for (tetO)7-CMV reporters lacking the upstream TK promoter when transfected with the tetR-KRAB construct). Additionally, the excellent repression of the tetR-KRAB is not compromised.

The reproducibility and efficacy of this system will be subject to the host cell line, integration site and investigator variances. Notably, BHK and Vero cell lines have been shown to display from 10 to 580 fold higher levels of induction than HeLa and PC12 lines using the (tetO)7-CMV operator and the tTA transactivating protein (Acklund-Berglund et al, "Efficacy Of Tetracycline-Controlled Gene Expression Is Influenced By Cell Type", *Biotechniques*, 18:196-200, 1995). Other tetR fusion proteins should also recognise the reporter vector of the present invention.

It is contemplated that the use of a regulator plasmid coding for the tetR-VP16 transactivating protein and the p(TK-(tetO)7-CMV-*lacZ*) reporter plasmid will result in an inverse pattern of induction relative to tetracycline.

Because the origins of the regulator plasmid include the KRAB repression domain, which is highly conserved amongst mammals, possible non-specific interaction with other *cis* sequences may occur. Such predicted interactions have been observed in the tTA system mentioned above.

Cell lines expressing tetR-KRAB and/or TK-(tetO)7-CMV-lacZ constructs using co-transfection with a neomycin expression vector, pMAMneo (Clonetech, Palo Alto, CA), have been constructed.

In another embodiment, it is envisioned that both promoters may be placed on the same "side" of the tetO operator sequence, i.e., both promoters may be upstream of the (tetO)7 operators or both promoters may be downstream of same. It is believed that the observed synergism of the above reporter construct would be achieved in such orientations as well.

Further, in another embodiment, it is expected that the reporter plasmid of the invention can be provided with more than two promoters.

Although the present invention has been described with reference to a construct having a TK promoter in conjunction with a CMV promoter, it will be apparent to a person skilled in the art that other promoter sequences could be used in the reporter plasmid of the invention. For example, one or both of the promoters could be substituted by Simian Virus 40 (SV-40) early or late promoter, Epstein-Barr Virus promoter or Baculovirus promoter. These promoters are all described in the literature and the readers attention is directed to, for example, S. Mongkolsuk, *Gene*, 70 (1988) 313; J. Sambrook et al., "Molecular Cloning" (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; and, S.E. Hasnain et al, *Gene*, 190(1) (1997) 113, the contents of each of which are incorporated herein by reference.

Further, it will be apparent to a person skilled in the art that the promoter-operator-promoter construct need not necessarily only be used in conjunction with the *lacZ* reporter gene. Other suitable reporter genes include the CAT-chloramphenicol transferase reporter (see, for example, J.H. Miller, "Experiments in Molecular Genetics", (1972) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), luciferase reporter (see, for example, J. Sambrook, "Molecular Cloning", (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), and the Green Fluorescent Protein (see, for example, M. Chalfie, *Photochem. Photobiol* (1995) 62(4), 651). The contents of these publications are incorporated herein by reference.

As is commonly known in the art, reporter plasmids such as described above, further include nucleotide sequences coding for any desired nucleic acid product. For example, such sequences may comprise RNA or sequences for structural proteins. By using a reporter gene as described, it is possible to confirm the expression of the desired nucleic acid sequences. As is also commonly known in the art, such reporter gene-desired gene construct would include an inter-ribosomal entry site (IRES) sequence separating the two elements. Such IRES sequence allows translation across two separate regions and would prevent any

transcriptional or translational interference that the reporter gene may exert on the desired gene.

It is envisioned that the on-off switch effect of the present invention can be used in many ways and not necessarily limited to a use with reporter genes. For example, the TK-(tetO)7-CMV construct of the present invention could be inserted into a nucleotide sequence including a sequence encoding any protein under investigation. In this way, it will be possible to determine the function of such protein by comparing the reaction of a cell or organism to the presence of or the absence of the protein. Alternatively, the reporter sequence of the invention could be placed upstream of any coding sequence regardless of function or nature. For instance, the end point may be a specific tRNA.

It is contemplated that the system of the present invention may be provided in the form of a kit. Such a kit would generally comprise at least two containers, the first container containing a polynucleotide molecule coding for the regulator plasmid including a gene for a transrepressor fusion protein comprising a tet repressor and a transcription silencer protein domain, the second container containing a polynucleotide molecule coding for the reporter plasmid including a tet operator sequence, at least two promoters and a sequence coding for a protein, wherein at least one of the at least two promoters is capable of being ligated to the gene sequence coding for a protein. Therefore, in a preferred embodiment, the kit would include one container having the regulator plasmid p(tetR-KRAB) and the other container would contain the reporter plasmid p(TK-(tetO)7-CMV-*lacZ*).

In another embodiment the operator sequence of the present invention can be replaced with any number of tetO sequences. This possibility is discussed by Gossen et al. (1992). It is envisaged that the presence of two promoters as discussed above would have a similar effect in a polynuleotide molecule having more or less than the heptameric tetO sequences.

In yet another embodiment, the reporter plasmid may be constructed with histidine tags instead of the *lacZ* gene. Such tags would facilitate the subsequent addition of any needed sequence.

Although the invention has been described with reference to certain specific embodiments, various modifications thereof will be apparent to those skilled in the art without departing from the spirit and scope of the invention as outlined in the claims appended hereto.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A polynucleotide molecule including a desired nucleic acid sequence operably linked to an operator sequence and at least two promoters.

2. The polynucleotide molecule of claim 1, wherein the operator sequence comprises a plurality of tetO sequences.

3. The polynucleotide molecule of claim 1 wherein operator sequence comprises a heptameric repeat of tetO sequences.

4. The polynucleotide molecule of claim 1, wherein at least one of the at least two promoters is positioned upstream of the operator sequence and at least one of the at least two promoters is positioned downstream of the operator sequence.

5. The polynucleotide molecule of claim 1, wherein the at least two promoters are selected from the group consisting of: a thymidine kinase promoter (TK) of Herpes Simplex Virus, a human cytomegalovirus promoter (CMV), a SV-40 early or late promoter, an Epstein-Barr Virus promoter (EBV) and a Baculovirus promoter.

6. The polynucleotide molecule of claim 1, wherein one of the at least two promoters is a human cytomegalovirus promoter (CMV).

7. The polynucleotide molecule of claim 1, wherein one of the at least two promoters is a thymidine kinase promoter (TK) of Herpes Simplex Virus.

8. The polynucleotide molecule of claim 1, wherein the operator sequence comprises a heptameric repeat of tetO sequences and wherein said tet operator sequence is operably linked to two promoter sequences.

9. The polynucleotide molecule of claim 8, wherein one of the two promoters is a thymidine kinase promoter (TK) of Herpes Simplex Virus and the other promoter is a human cytomegalovirus promoter (CMV).

10. The polynucleotide molecule of claim 9 wherein the TK promoter is positioned upstream of the tet operator sequence and the CMV promoter is positioned downstream of the tet operator sequence.

11. The polynucleotide molecule of claim 1 wherein the operator sequence comprises a heptameric repeat of the tet operator and is positioned between an upstream TK promoter and a downstream CMV promoter and wherein said desired nucleic acid sequence is positioned downstream of the CMV promoter.

12. The polynucleotide molecule of claim 1, wherein the desired nucleic acid sequence encodes a protein or RNA.

13. The polynucleotide molecule of claim 1, wherein the desired nucleic acid sequence includes a gene for a reporter protein, said protein being selected from the group consisting of: bacterial β-galactosidase, CAT-chloramphenicol transferase, luciferase, and Green Fluorescent Protein.

14. The polynucleotide of claim 1 wherein said desired nucleic acid sequence includes a gene for a reporter protein and a gene for a desired cellular product.

15. A vector comprising the polynucleotide molecule according to claim 1.

16. The vector of claim 15 wherein said vector comprises a λ phage transfected with said polynucleotide molecule.

17. A eukaryotic cell transfected with:

a first polynucleotide molecule including a desired nucleic acid sequence operably linked to a tet operator sequence and at least two promoters; and,

a second polynucleotide molecule coding for a transrepressor fusion protein comprising a tet repressor and a transcription silencer protein.

18. The cell of claim 17 wherein said tet operator sequence comprises a heptameric repeat of the tet operator.

19. The cell of claim 17, wherein the at least two promoters are selected from the group consisting of a thymidine kinase promoter of Herpes Simplex Virus, a human cytomegalovirus promoter, a SV-40 early or late promoter, a Epstein-Barr Virus promoter and a Baculovirus promoter.

20. The cell of claim 17, wherein one of the at least two promoters is a human cytomegalovirus promoter.

21. The cell of claim 17, wherein one of the at least two promoters is a thymidine kinase promoter of Herpes Simplex Virus.

22. The cell of claim 17, wherein the desired nucleic acid sequence of the first polynucleotide molecule, includes a gene for a reporter protein, said protein being selected from the group consisting of: bacterial β-galactosidase, CAT-chloramphenicol transferase, luciferase, and Green Fluorescent Protein.

23. The cell of claim 17, wherein the tet operator sequence is downstream of one of the at least two promoters and upstream of one of the at least two promoters.

24. The cell of claim 17, wherein said first polynucleotide molecule includes two promoters and wherein one of the promoters is a thymidine kinase (TK) promoter of Herpes Simplex Virus and the other promoter is a human cytomegalovirus (CMV) promoter.

25. The cell of claim 25, wherein the TK promoter is positioned upstream of the tet operator sequence and the CMV promoter is positioned downstream of the tet operator sequence and wherein said desired nucleic acid sequence is positioned downstream of the CMV promoter.

26. The cell of claim 17, wherein the second polynucleotide molecule includes a gene for a tet repressor protein (tetR) and the Kruppel-associated box domain (KRAB) of Koxl Zn-finger protein.

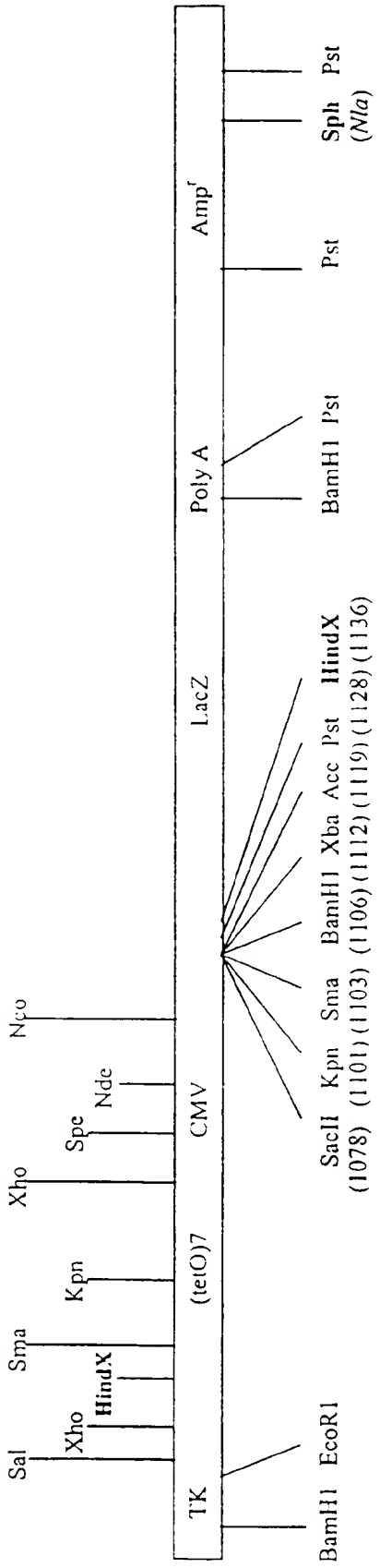
27. A method to induce expression of the desired nucleic acid sequence of the first polynucleotide of claim 27, the method comprising cultivating the cell of claim 27 in a medium comprising tetracycline or a tetracycline analogue.

28. A kit comprising at least two containers wherein the first container contains a first polynucleotide molecule including a desired nucleic acid sequence operably linked to a tet operator sequence and at least two promoters and wherein the second container contains a second polynucleotide molecule including a gene for a tet repressor protein (tetR) and the Kruppel-associated box domain (KRAB) of Koxl Zn-finger protein.

29. The kit of claim 29, wherein said first polynucleotide molecule includes two promoters and wherein one promoter is provided on each side of the tet operator sequence.

30. The kit of claim 30 wherein the upstream promoter is TK and the downstream promoter is CMV.

31. The kit of claim 31 wherein said desired nucleic acid sequence of the first polynucleotide molecule is positioned downstream of the CMV promoter and includes a gene for a reporter protein.


TK
(tetO)7
CMV
LacZ
Poly A
Amp^r
Sal
Xho
HindX
Sma
Kpn
Xho
Spe
Nde
Nco
BamH1
EcoR1
SacII (1078)
Kpn (1101)
Sma (1103)
BamH1 (1106)
Xba (1112)
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Pst (1128)
HindX (1136)
BamH1
Pst
Pst
Sph (Nla)
Pst

Figure 1

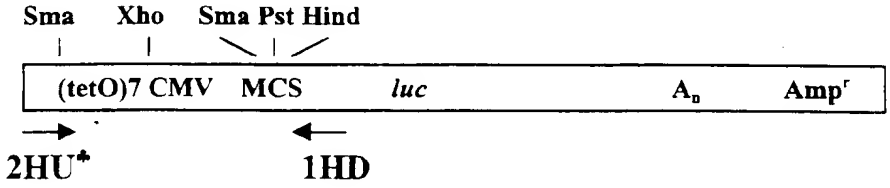

Figure 2a

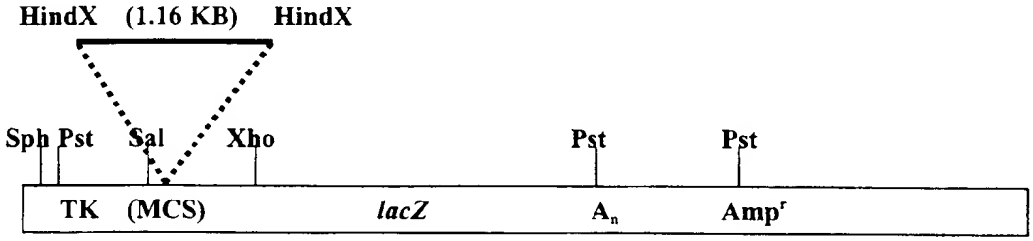

Figure 2b

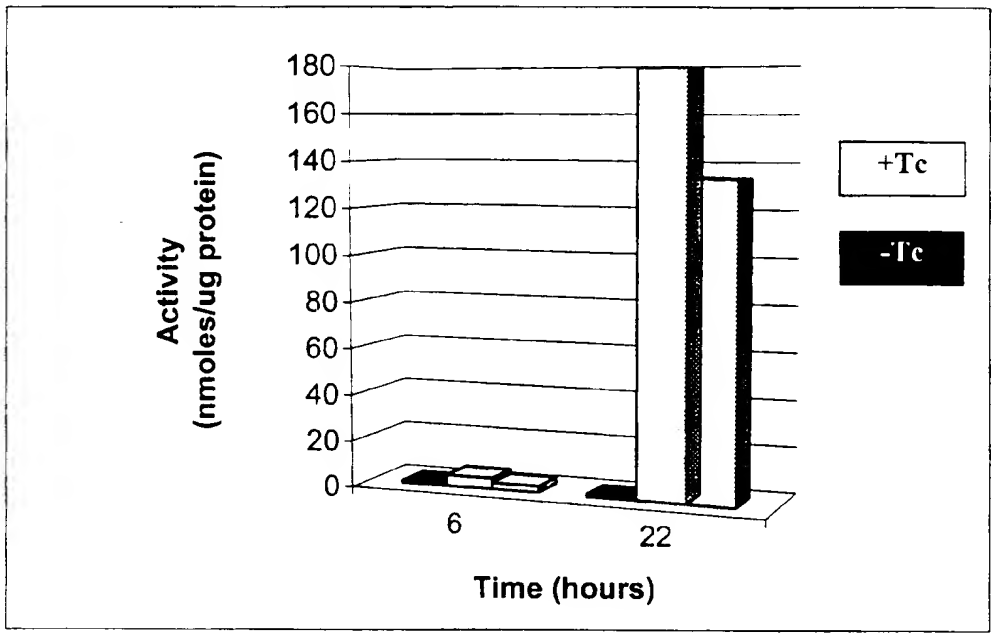
180
160
140
120
100
80
60
40
20
0
Activity
(nmoles/ug protein)
6
22
Time (hours)
+Tc
-Tc

Figure 3

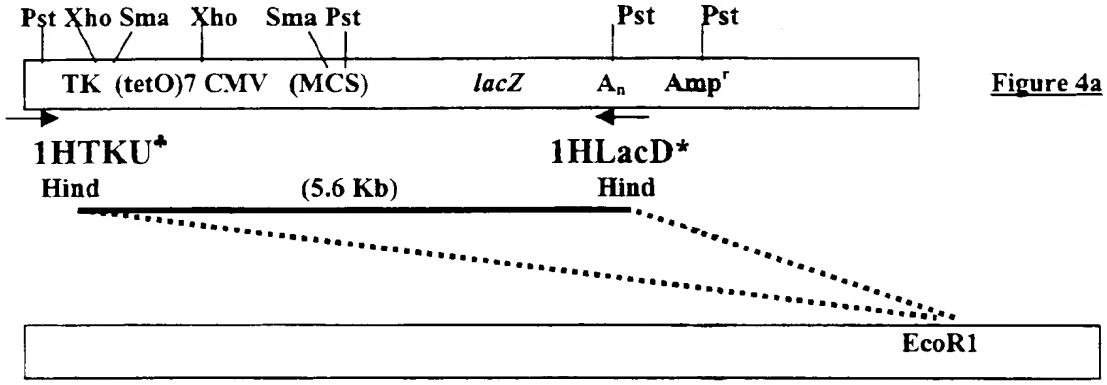

Figure 4a

Figure 4b

5' ACG AGG CCC TTT AAG CTT CAA GAA TTC CTC 3'

Figure 5

5' CAG TAC CGG AAT GCC AAG CTT GCA 3'

Figure 6

5' GAA AGT CCC CAA GCT TCC CAG CAG GCA GA 3'

Figure 7

5' GGG CAG CCT AAG CTT GGA ATG TCC TCT C 3'

Figure 8

5' AG CTT CAA GAA AGC TTC GAG CCG GGT ACC 3'

Figure 11

5' CCG GGT ACC GAG CTC GAA TTC G 3'

Figure 13

5' CAG CAG GCA GAA GTA TGC AAA GC 3'

Figure 14

Figure 9

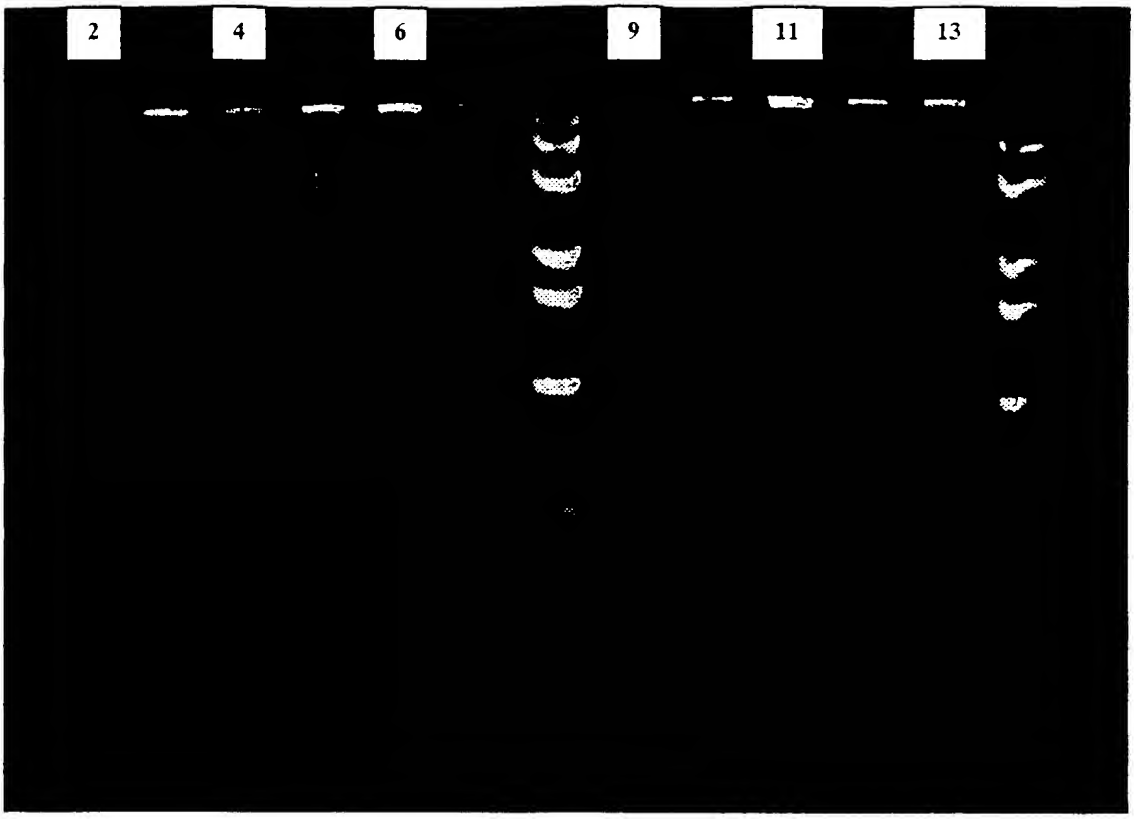

Figure 10

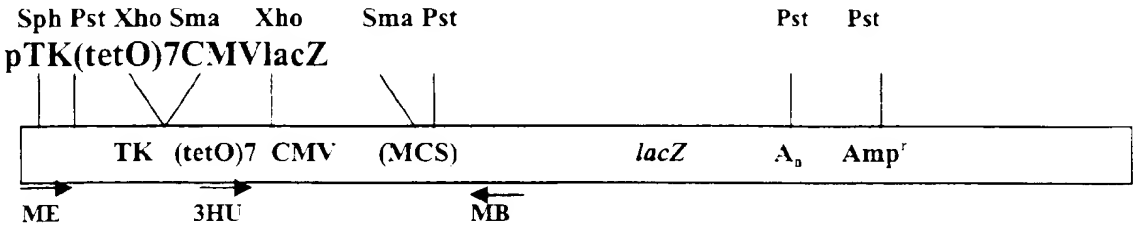

Figure 12

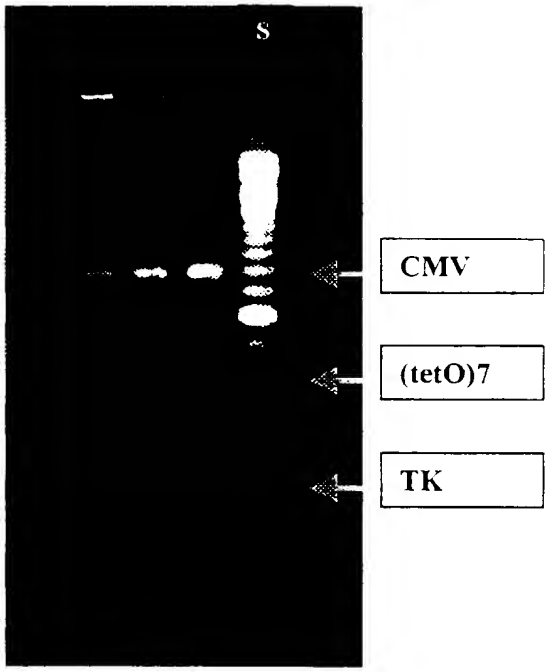

Figure 15

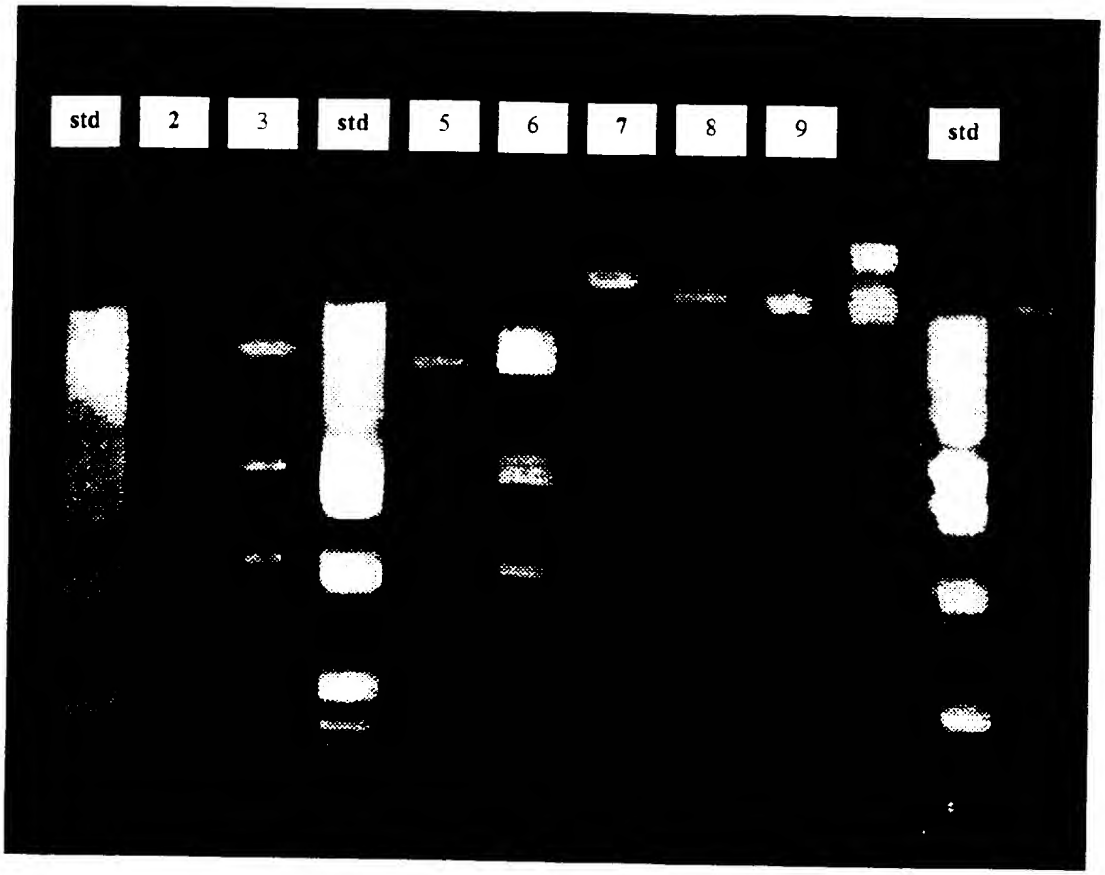

Figure 16

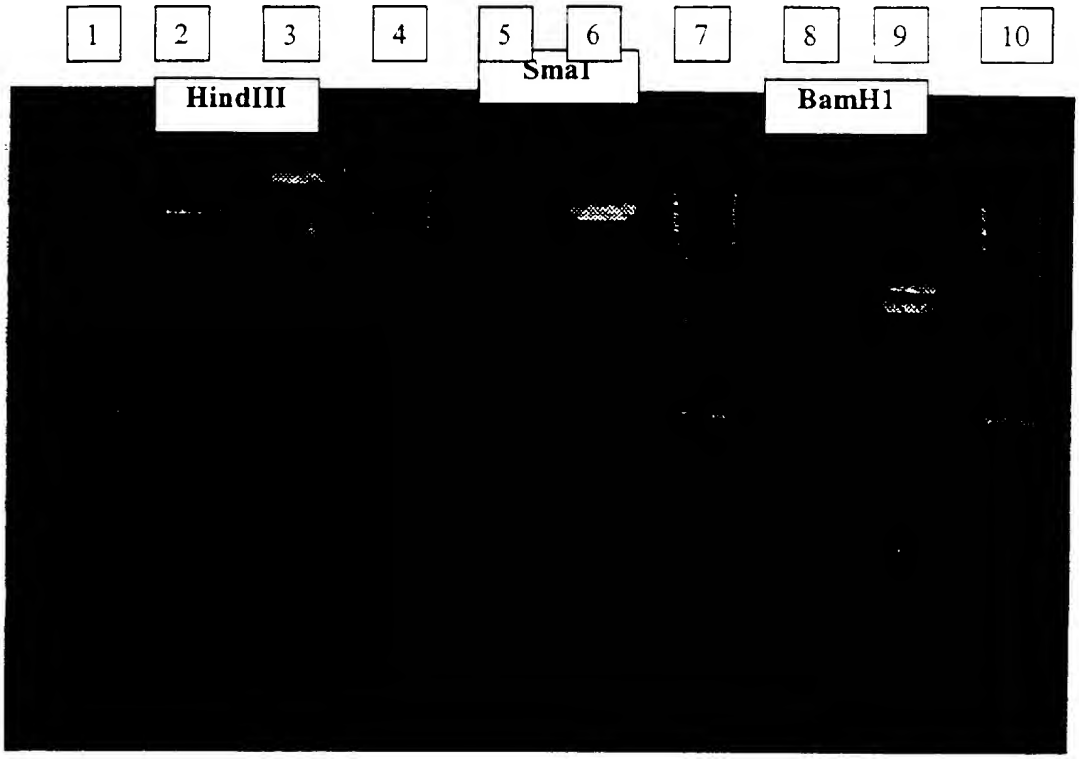

Figure 17

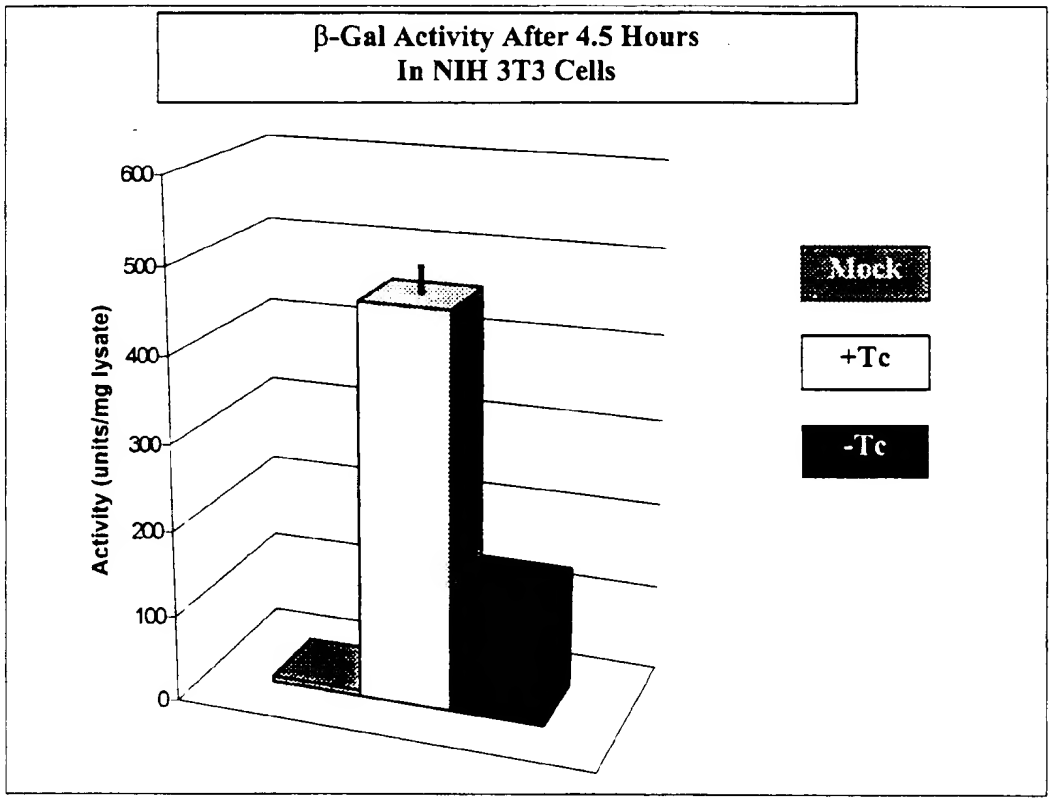
β-Gal Activity After 4.5 Hours
In NIH 3T3 Cells
Activity (units/mg lysate)
600
500
400
300
200
100
0
Mock
+Tc
-Tc

Figure 18

SEQUENCE LISTINGS

1) GENERAL INFORMATION

i) APPLICANTS:

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ii) TITLE OF INVENTION: SYSTEM AND METHOD FOR REGULATION OF GENE EXPRESSION

iii) NUMBER OF SEQUENCES: 7

iv) CORRESPONDENCE ADDRESS:

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v) COMPUTER READABLE FORM:

a) COMPUTER: PC

b) OPERATING SYSTEM: Windows

c) SOFTWARE: DOS Editor

vi) CURRENT APPLICATION DATA:

a) APPLICATION NUMBER:

b) FILING DATE: November 16, 1998

c) CLASSIFICATION:

vii) PRIOR APPLICATION DATA:

a) APPLICATION NUMBER: US 08/970,315

b) FILING DATE: November 14, 1997

c) CLASSIFICATION: 435

viii) PATENT AGENT INFORMATION:

a) NAME: Santosh K. Chari

b) REFERENCE NUMBER: 8700151-0003

2) INFORMATION FOR SEQ ID NO:1

i) SEQUENCE CHARACTERISTICS:

a) LENGTH: 30 base pairs

b) TYPE: Nucleic Acid

c) STRANDEDNESS: Both

d) TOPOLOGY: Both

ii) SEQUENCE DESCRIPTION: SEQ ID NO:1

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2) INFORMATION FOR SEQ ID NO:2

i) SEQUENCE CHARACTERISTICS:

a) LENGTH: 24 base pairs

b) TYPE: Nucleic Acid

c) STRANDEDNESS: Both

d) TOPOLOGY: Both

ii) SEQUENCE DESCRIPTION: SEQ ID NO:2

CAG TAC CGG AAT GCC AAG CTT GCA 24

2) INFORMATION FOR SEQ ID NO:3

i) SEQUENCE CHARACTERISTICS:

a) LENGTH: 29 base pairs

b) TYPE: Nucleic Acid

c) STRANDEDNESS: Both

d) TOPOLOGY: Both

ii) SEQUENCE DESCRIPTION: SEQ ID NO:3

GAA AGT CCC CAA GCT TCC CAG CAG GCA GA 29

2) INFORMATION FOR SEQ ID NO:4

i) SEQUENCE CHARACTERISTICS:

a) LENGTH: 28 base pairs

b) TYPE: Nucleic Acid

c) STRANDEDNESS: Both

d) TOPOLOGY: Both

ii) SEQUENCE DESCRIPTION: SEQ ID NO:4

GGG CAG CCT AAG CTT GGA ATG TCC TCT C 28

2) INFORMATION FOR SEQ ID NO:5

i) SEQUENCE CHARACTERISTICS:

a) LENGTH: 29 base pairs

b) TYPE: Nucleic Acid

c) STRANDEDNESS: Both

d) TOPOLOGY: Both

ii) SEQUENCE DESCRIPTION: SEQ ID NO:5

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2) INFORMATION FOR SEQ ID NO:6

i) SEQUENCE CHARACTERISTICS:

a) LENGTH: 22 base pairs

b) TYPE: Nucleic Acid

c) STRANDEDNESS: Both

d) TOPOLOGY: Both

ii) SEQUENCE DESCRIPTION: SEQ ID NO:6

CCG GGT ACC GAG CTC GAA TTC G 22

2) INFORMATION FOR SEQ ID NO:7

i) SEQUENCE CHARACTERISTICS:

a) LENGTH: 23 base pairs

b) TYPE: Nucleic Acid

c) STRANDEDNESS: Both

d) TOPOLOGY: Both

ii) SEQUENCE DESCRIPTION: SEQ ID NO:7

CAG CAG GCA GAA GTA TGC AAA GC 23

INTERNATIONAL SEARCH REPORT

Internati .l Application No
PCT/CA 98/01056

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 C12N15/67 C12N15/63 C12N5/10 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SWIGER, ROY R. ET AL: "A beta-galactosidase reporter vector for mammalian cell lines." ENVIRONMENTAL AND MOLECULAR MUTAGENESIS, (1998) VOL. 31, NO. SUPPL. 29, PP. 28. MEETING INFO.: 29TH ANNUAL MEETING OF THE ENVIRONMENTAL MUTAGEN SOCIETY ANAHEIM, CALIFORNIA, USA MARCH 21-26, 1998 ENVIRONMENTAL MUTAGEN SOCIETY. ISSN: 0893-6692., XP002099424 Abstract no. P6 see abstract --- -/--	1-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 April 1999	22/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internati . Application No
PCT/CA 98/01056

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No
A	BARON U ET AL: "CO-REGULATION OF TWO GENE ACTIVITIES BY TETRACYCLINE VIA A BIDIRECTIONAL PROMOTER" NUCLEIC ACIDS RESEARCH, vol. 23, no. 17, 11 September 1995, page 3605/3606 XP000775822 see the whole document	1-31
A	DEUSCHLE U ET AL: "TETRACYCLINE-REVERSIBLE SILENCING OF EUKARYOTIC PROMOTERS" MOLECULAR AND CELLULAR BIOLOGY, vol. 15, no. 4, April 1995, pages 1907-1914, XP000601654 cited in the application see the whole document	1-31
A	WO 97 35992 A (VICAL INC) 2 October 1997 see the whole document	1-31
A	WO 96 40892 A (BASF AG) 19 December 1996 see the whole document	1-31
A	WO 96 01313 A (BUJARD HERMANN ;GOSSEN MANFRED (US)) 18 January 1996 see the whole document	1-31
A	WO 94 04672 A (DNX CORP ;BYRNE GUERARD (US)) 3 March 1994 see the whole document	1-31
A	US 5 464 758 A (GOSSEN MANFRED ET AL) 7 November 1995 cited in the application see the whole document	1-31

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat I Application No
PCT/CA 98/01056

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9735992	A	02-10-1997	NONE		
WO 9640892	A	19-12-1996	US	5589362 A	31-12-1996
WO 9601313	A	18-01-1996	US	5654168 A	05-08-1997
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			EP	0665883 A	09-08-1995
US 5464758	A	07-11-1995	US	5789156 A	04-08-1998
			US	5589362 A	31-12-1996
			US	5814618 A	29-09-1998
			US	5866755 A	02-02-1999